The Effects of Urbanization on Dermal Corticosterone of Eastern Red-Backed Salamanders, *Plethodon cinereus* in Ohio

Mackenzie Brown, School of Environment and Natural Resources, The Ohio State University, 2021 Coffey Rd, Columbus, OH 43201

Introduction

We are currently amidst a 6th mass extinction event precipitated by anthropogenic alterations of the environment (Ceballos et. al 2017). Urbanization is among the most detrimental types of habitat modifications (Hayes et. al 2010) and by 2050, the world is projected to have a population of 10 billion people and of those, 66% are expected to be living in urban areas (Leeson 2018). As people move into and expand urban areas, natural areas are often converted and modified to meet human needs, to the detriment of wildlife. There have been declines across all classes of vertebrates, however amphibians have suffered the greatest declines among vertebrates (Stuart et al. 2004, Hoffmann et al. 2010). Approximately 70% of global amphibian populations are declining as a result of anthropogenic causes (Hayes et. al 2010), and the current extinction rates of amphibians is estimated to be 211 times greater than the calculated background extinction rate (Mccallum 2007). As amphibian populations continue to decline, researchers have investigated different possible causes and have determined that a combination of climate change, increase in environmental pollutants, and prevalence of invasive species have all affected amphibians (Hayes et. al 2010). However, habitat loss and modification are the leading cause of declines because it increases susceptibility and exposure to disease and

chemicals, increases the probability of encountering invasive species, alters climate and microclimates, and ultimately leads to decreased recruitment and population size in amphibian populations (Hayes et. al 2010). Urbanization also increases the surface area of heat absorbing surfaces, which often come in the form of impermeable surfaces (Kaiser et. al 2016). Heat absorbing surfaces can increase ambient temperatures, decrease relative humidity, reduce evapotranspiration, increase runoff, decrease land filtration of water, and increase high discharge rates of water into stream systems (Kaiser et. al 2016). Many amphibians rely on moist and cool conditions for survival (Peterman & Semlitsch 2013; Gade & Peterman 2019) and the combined decrease of humidity and increase of temperature may lead to highly unfavorable conditions for amphibians. The decline or loss of amphibians could be catastrophic to ecosystem function, as amphibians provide many benefits to ecosystems including nutrient cycling, pest controls, decreasing disease transmission, and altering rates of leaf litter decomposition (Walton et. al 2006; Hocking and Babbit 2014) making their declines potentially catastrophic to total ecosystem function.

Amphibians have strict physiological needs that are largely driven by attributes of the habitat (integrity, area, patch size) (Peterman & Semlitsch 2013). As habitat is altered, degraded, and fragmented changes to surface soil temperatures and moisture can occur. If basic conditions for reproduction and foraging are not met, alteration in behavior or physiological processes may be observed. When an amphibian experiences a stressor such as predation attempts, disease, lack of resources, or unsuitable abiotic conditions, a hormonal response occurs that causes the brain to trigger the release of corticotrophin-releasing hormone from the hypothalamus-pituitary-interrenal axis (Rollins-Smith 2017, Santymire et. al 2018). The anterior pituitary then releases adrenocorticotropic-releasing hormone into the bloodstream, where it travels to the

adrenal/interrenal gland leading to the release of glucocorticoid hormones, which include corticosterone (CORT), from the adrenal cortex (Reeder and Kramer 2005). When released, CORT helps amphibians respond to a stressor appropriately by suppressing acutely nonessential functions such as digestion, reproduction, and growth, thereby freeing energy for vital processes such as muscle metabolism (Romero & Reed 2008). However, when released over long time periods when stressors occur chronically, increased levels of CORT can inhibit immune response, hinder growth, halt reproduction, disrupt cell messengers, and lead to protein loss (Sapolsky et. al 2000). Chronic elevation of CORT levels can ultimately lead to decreased fitness (Millikin et. al 2019), and thus CORT can be an important measure of physiological stress in response to human disturbances on amphibians (Dantzer et. al 2014).

As amphibian populations are increasingly threatened by habitat loss and urbanization, this has lead to an increase in our research and understanding of the physiological responses of amphibians to these pressures. For example, yellow-bellied toads, *Bombina variegata*, in areas with high vehicle traffic had suppressed levels of CORT, decreased fitness, and decreased annual survival rates (Cayuela et al. 2015). Further, toads living in degraded habitat (alteration of terrestrial habitat) had high levels of nutritional stress and decreased abundance due to a lower abundance and quality of food (Cayuela et al. 2015). However, Janin et al. (2012) found that the common toad, *Bufo bufo*, had higher CORT levels in disturbed, plowed substrate than forest or meadow substrate. Similar to Cayuela et al. (2015), in populations of spotted salamanders, *Ambystoma maculatum*, Millikin et. al (2019) found that spotted salamanders residing within highly disturbed, small forest patches had depressed levels of CORT. This could be attributed to chronic stress causing CORT hormone production to be reduced or suppressed (Homan et. al 2003). Homan et. al (2003) found that chronic stress led to depressed baseline levels of CORT

and hampered ability to release CORT in response to a stressor for populations of spotted salamanders. Homan et. al (2003) also found that salamanders that had to cross over pavement rather than forested areas to reach breeding pools had an increase in CORT levels, suggesting that acute stressors may cause a spike in CORT concentrations.

Wildlife in urban areas are more likely to experience disturbances to their habitats, raising the likelihood of experiencing chronic stress. Amphibians are sensitive to habitat change during both the terrestrial and aquatic life stages. In the aquatic stage, eggs and larvae amphibians need to remain in water for several months and occasionally up to a year (Vos et al. 2001). In the terrestrial stage, dispersal from natal sites and seasonal migrations to and from breeding habitats increases vulnerability to environmental conditions that can be altered by humans. (Vos et al. 2001; Semlitsch & Bodie 2003). The quality of environment and habitat around these sites is likely to be an important driver for urban amphibian viability (Semlitsch & Bodie 2003). In a study of 20 European cities, amphibian populations were seen to decline in all cities, with the complete disappearance of some species altogether (Kaczmarek et al. 2014; Konowalik et al. 2020; Najbar et al. 2005). Two of those cities were studied for long-term population trends and there was an observed decrease of 20% in one city and 40% in the other of historical amphibian breeding sites due to urbanization (Najbar et al. 2005; Kaczmarek et al. 2014). Due to their habitat requirements, most amphibians cannot persist in small habitat patches in urban environments, however, eastern red-backed salamanders, *Plethodon cinereus,* are among the few able to survive in more disturbed, isolated, urban habitat patches (Riedel et al. 2008).

Eastern red-backed salamanders are the most abundant terrestrial vertebrate in North American hardwood forests and can comprise up to 99% of vertebrate biomass in some terrestrial ecosystems (Burton & Likens 1975a). Eastern red-backed salamanders have a large

geographic range spanning the eastern U.S. to Wisconsin and Minnesota and North to Quebec and Nova Scotia (Lannoo 2004). Terrestrial salamanders, such as the eastern red-backed salamander, serve many key ecosystem functions including slowing leaf litter decomposition by acting as an important predator for invertebrates (Wyman 1998), altering soil dynamics (increase aeration, decrease compaction, opening of pore space, etc.), supplying key nutrients for tertiary consumers (Davic & Welsh Jr. 2004), and increasing carbon sequestration through predation of lower trophic level species that feed on organic matter (Anthony et al. 2017). Understanding how urbanization affects eastern red-backed salamanders is vital to preserving these functions. It has been shown that some species of salamanders respond physiologically to environmental changes, suggesting a link between habitat quality and physiological stress response that could be crucial for evaluating the effects of habitat alterations. Novarro et. al (2018) found that there was a significant difference in CORT levels among eastern red-backed salamanders at different latitudes and at temperatures across their range. However, there is a knowledge gap in how habitat disturbance and urbanization affect physiological stress in these salamanders.

The goal of the present study is to determine how urbanization affects the physiological stress of eastern red-backed salamanders. The stress of habitat loss and urbanization may lead to increased levels of CORT, the primary stress hormone released by most amphibians (Homan et. al 2003). Because urban habitat patches are smaller, warmer, and tend to be more disturbed, we predict that eastern red-backed salamanders occupying urban forest patches will be more chronically stressed, which will be evident in suppressed CORT levels when compared to salamanders occupying non-urban forest patches.

Methods

Study Sites

We sampled salamanders from six forested patches in the Columbus, OH region from 6 October to 16 October 2020. Three urban and three non-urban park locations were selected based on the amount of impervious surface and percent of forested area (NLDC 2016 Landcover 2021) within a 1 km and 5 km buffer area of collection sites (Figure 1; Figure 2). We downloaded a landcover raster layer from the 2016 National Landcover Database and reclassified it into 6 landcover categories: (1) Urban, which combined all "developed" categories, (2) Forest, which combined all "forest" categories, (3) Grasslands, which combined all "shrubland" and "herbaceous" categories, (4) Agriculture, which combined and "Planted/Cultivated" categories, (5) Wetland, which combined the two "wetlands" categories, and (6) Water. Using a centroid that was hand designated as the central location of sampling of all individual salamanders at each park, we characterized the percent of each landcover class within a 1 km and 5 km circular buffer around each centroid (Figure 2) using the *landscapemetrics* package in R (Hesselbarth et al. 2019). Urban sites had a mean area of 19.43 ha while nonurban sites had a mean area of 174.37 ha (Table 1).

Field Methods

Our study uses a relatively novel method to measure dermal corticosterone (hereafter dCORT) collection using dermal swabs which is non-invasive and non-lethal for salamanders (Santymire et. al 2018). In addition, dermal swabs can be easily and rapidly obtained on the same individual. In the past, assessments of CORT required collection of blood, which is often lethal for salamanders, or a water-borne assays, which is time-intensive and may affect the interpretation of baseline CORT (Navarro et. al 2018). Preliminary assessments of this method in the lab have demonstrated its validity in a congeneric species (M. Gade, unpublished data). This

method could be used for most amphibian species because the original validation study tested their method on multiple terrestrial, semi-aquatic, and aquatic species of amphibians (Santymire et. al 2018). The present study is the first of its kind to use dCORT to evaluate physiological stress in response to urbanization in eastern red-backed salamanders at a population level.

On each sampling day, we collected dermal swabs on 30 individuals at a single site. Salamanders were located by flipping surface cover such as woody debris and rocks. Large logs and rocks were avoided to help minimize the disturbance of important microhabitat. When found, salamanders were placed in a sterile, plastic container (12.6 x 10.5 x 9.5 cm) and swabbed with a sterile wooden-shafted cotton swab from the posterior of the head to the anterior of the tail on the anterior surface of the body approximately ten times while rotating the cotton swab. Swabs were placed into individually labeled microcentrifuge tubes prefilled with 2 mL of 90% ethanol. Snout-vent length to the nearest 0.1 mm and sex of the salamander were recorded, and the salamander was released at its capture location within 15 minutes of initial capture. Nitrile gloves were worn the entire time the salamanders were being handled. We recorded the location of each salamander captured using a handheld GPS (Garmin model # 010-01199-10), air temperature, humidity, and soil temperature were measured using a kestrel and soil probe, respectively. All salamanders were swabbed within three minutes of initial capture, a time frame that has been shown to reflect the baseline, pre-capture dCORT levels (Romero & Reed 2008). Samples were stored in the lab at 5 °C until processing occurred.

Laboratory Methods

The hormone extraction process was completed using the methods initially described by Santymire et. al (2018). The hormone extraction process methodology was modified, and we validated the use of our chosen Enzyme Immunoassay (EIA) using assay parallelism. To confirm assay parallelism, we compared the slopes of serial dilutions of EIA assay kit standards (Neogen, Lexington, KY) to serial dilutions from swabs taken from nine individuals. Using ANCOVA, we found no difference between standards and unknowns in the slope of percent of the assay conjugate bound to plate antibody (relative binding) compared to log transformed dCORT concentration (group*concentration interaction: $F= 0.148$, $P= 0.704$, Supplemental Fig. 1).

For each assay, thirty-nine samples were randomly selected to ensure randomization of samples from different sites. Each tube containing a swab was vortexed for 45 seconds before transferring the ethanol in from a microcentrifuge tube into a drying tube. Each swab was tapped against the side of the drying tube to express any remaining ethanol. The drying tubes were then submerged in a 50°C shaking water bath and dried under nitrogen air until the ethanol was evaporated from the tubes (~1.5 hours). After the drying process was complete, the samples were reconstituted using 2000 µL of buffer solution (provided with ELISA hormone assay kit) and vortexed for 30 seconds. The samples were then run in duplicate on commercial EIA from Neogen Corporations. The samples were measured against a standard curve following the Neogen protocols and materials. Samples from every site were randomly analyzed across 6 assays from 18 November 2020 to 28 January 2021. All CORT measures were standardized by an individual's snout-vent length (SVL) by dividing dCORT by SVL to obtain the concentration of dCORT (pg/swab).

Statistical Analyses

We used a linear mixed effect model in a Bayesian framework with urban status as the fixed effect and site as the random effect using the *bmrs* package in R (Bürkner 2017). Urban status was fit as a continuous proportion of buffer with a buffer radius of 1 km and 5 km. We fit the model with four MCMC chains for 3000 iterations after a burn-in phase of 1500, thinned at a rate of 1. The model used a Gaussian distribution with uninformative flat priors. Model fit was assessed by inspection of MCMC chain mixing and the Gelman-Rubin statistic (Rhat <1.1). Posterior predictive plots were plotted and inspected. A region of practical equivalence (ROPE) test was then run as an equivalence test of significance to show the level of credibility of the parameter values. The ROPE analysis determines the proportion of the posterior distribution for the estimated parameter(s) that is within the Region Of Practical Equivalence. This region is statistically defined as being the range of values that do not meaningfully differ from zero (i.e. no effect). If little to none of the posterior distribution is within ROPE, then the estimated effect is interpreted to have a meaningful effect. The *Bayesian First Aid* (Baath 2013) package was used to calculate the mean SVL for urban and non-urban sites and run a t-test to see if there was a significant difference in values.

Results

On average, the measured dermal CORT concentrations in non-urban areas (545.6 pg/swab ± 153.3) were higher than that of urban areas (377.0 pg/swab ± 64.8) (Table 2, Figure 3). On average, dCORT was estimated to be significantly less at urban sites (beta = -1.92, 95% CRI: -4.72, 0.82) with 93.3% of the posterior distribution corroborating this trend. 6.74% of the posterior distribution was within the ROPE, suggesting a meaningful effect on dCORT response (Piironen and Vehtari 2017). Visual inspection of posterior predictive plots indicated that our model fit the data well.

There were clear differences between urban and non-urban sites in terms of the amount of forest cover and urban development surrounding sample sites. Urban sites had an average of 7.2% forest cover within a 1-km buffer and an average of 3.4% forest cover within a 5-km buffer. Non-urban sites had an average of 79.5% forest cover within a 1-km buffer and 35.4% forest cover within a 5-km buffer. The urban sites had 90.8% urban land class cover within 1-km and 94.2% within 5-km. Non-urban sites had 6.3% urban land class cover within 1-km and 22.9% within 5-km (Figure 2). The mean SVL of animals in urban areas (3.52 ±0.55 cm) was significantly smaller than the SVL of animals in non-urban (3.79 ± 0.48 cm) with the difference of means (-0.27, CRI: -0.44, -0.11).

Discussion

We found that eastern red-backed salamanders in forested patches within an urban matrix had significantly lower baseline levels of dCORT than salamanders from non-urban sites. Because eastern red-backed salamanders are lungless, they require microhabitats with cool temperatures and high moisture levels to facilitate respiration though their skin (Spotila 1972). Salamanders are more abundant in older stand aged forests with increased downed woody debris, deep soils, and abundant rocks (Hyde & Simmons 2001). Urbanized areas typically do not have an abundance of these characteristics and are thus likely less suitable environments for eastern red-backed salamanders. Our results are consistent with those of Homan et. al (2003), who found chronic stressors from urbanization led to depressed baseline levels of stress hormone in spotted salamanders. Depressed levels of baseline dCORT could be an effect of chronic stress, which leads to both a decreased stress response at baseline levels and when faced with an acute stressor (Homan et. al 2003). To test whether dCORT increase is suppressed after acute stress exposure, (i.e. that eastern red-backed salamanders have a muted stress response), we suggest a post-

handling stress response experiment at urban and rural sites. If urban salamanders had an absent or low stress response in response to an acute stressor it would indicate a disturbance in the regular dCORT cycle, which is a direct effect of chronic stress (Homan et. al 2003). The lower baseline dCORT in urban salamanders could also suggest that urbanization at a broad scale does not affect the dCORT levels of salamanders since salamanders operate at small scale, but instead that fine scale microclimate and microhabitat may be the driving factors for environmental stress. These microhabitats and microclimates are ultimately altered by human activity such as urbanization. We found that on average, the urban sites sampled had higher soil moisture levels, but also higher soil temperatures (Table 1, Table 2). This does not provide much clarity as to what is driving the stress response in the ecosystems studied. Higher soil moisture would provide a less stressful habitat for salamanders, but higher soil temperatures would provide a more stressful habitat. To examine these differences, local weather patterns over the 10-day collection time could be analyzed, however additional research is needed to corroborate these findings. Additionally, further research needs to be conducted to see if the effect we saw of urbanization on physiological stress is through broad or fine scale impacts of the surrounding environment.

Our study showed that salamanders in urban sites had a significantly shorter SVL than salamanders in non-urban sites. It is possible that this difference in length could be a result of energy budgeting. If eastern red backed salamanders in urban sites are experiencing chronic stress, they may have to reallocate energy away from foraging, reproduction, etc. and put that energy towards maintenance (metabolism, acutely essential activities) (Homyack et. al 2011). Salamanders could also be shortening their normal surface-active season to avoid the harshest conditions on surface substrate, which would greatly curtail their foraging and reproduction ability, as eastern red-backed salamanders forage and reproduce above ground. An energetics

study of eastern red-backed salamanders showed that eastern red-backed salamanders in areas with removed overstory cover (poor quality habitat) allocated 33% more calories towards basic maintenance than eastern red-back salamanders in the control area (ideal habitat) (Homyack et. al 2011). Areas with poor habitat, such as removed overstory cover, are subjected to higher temperatures and drier conditions. Increased temperatures have been shown to cause an energy deficit at temperatures of 20°C or greater in eastern red-backed salamanders due to decreased energy assimilation from prey (Merchant 1970). The decreased ability to forage, grow, and reproduce is inherently stressful and if salamanders are consistently existing in those habitat patches, we could expect depressed levels of dCORT to be caused from chronic stress.

Although we analyzed our sites on a continuum of urbanization rather than a binary category, a greater breadth of site urbanization levels would offer valuable information and would be an interesting way to expand the work in this study. Further research should additionally explore specific parts of urbanization (proximity to roads, noise/air pollution, etc.) that are the most influential in suppressed dCORT responses in urban salamanders. Another direction for future research is to look at the local environment and microclimate the salamanders are surrounded by. This could include but not be limited to factors such as soil moisture, temperature, humidity, and percent canopy cover (Appendix Table 1) (Appendix Table 2). Although our sample size of 30 salamanders per site has been deemed adequate in previous studies that use 15 – 25 salamanders (Homan et. al 2002; Gabor et. al 2018; Novarro et al. 2018), a larger sample size would provide further rigor and confidence in looking at each of these effects.

The novel method of dermal swabbing can save both time and money. Other methods of stress hormone analysis involve either a blood sample or a water-borne assay. The blood draw method is not always plausible for studying salamanders because it can be lethal due to their low

blood volume compared to other animals. When the blood sample is taken, often the salamanders do not have enough blood left to survive due to their small size. Dermal swabbing is non-lethal which would eliminate the need to sacrifice animals, making it possible to study endangered and threatened species that cannot be sacrificed or removed from the field. Water borne assays measure the amount of hormone in fecal matter released into the water over a multiple week period. This method is time intensive and requires skilled labor which increases expenses. Dermal swabbing offers a unique solution because it does not require skilled workers to be hired like other stress assay methods, and training can be relatively short (an hour). In addition, samples can be taken in situ, reducing the inherent stress of moving animals to the lab to obtain measurements. This could save countless lab hours as the samples are ready to be run when they are brought back to the lab.

As urban areas continue to expand globally, it is vital to understand how urbanization is affecting amphibians. Amphibians are broadly threatened by climate change, and as habitat and land use continue to be altered, the environment is becoming more hostile for amphibian survival. Declining amphibian populations, in addition to the multiple stressors of climate change, habitat loss, and habitat degradation, will continue to increase in the coming years, but by understanding the physiological responses of amphibians to these stressors, we might begin to better understand the mechanisms by which populations will decline.

References

Anthony, C., Hickerson, C.M., & Walton, M. (2017). Eastern red-backed salamanders regulate top down effects in a temperate forest-floor community. *Faculty bibliography, 33.*

Bååth, R. (2014). Bayesian first aid: A package that implements bayesian alternatives to the classical *.test functions in R. In the proceedings of *UseR! 2014 - the International R User Conference.*

Best, M. L., & Welsh Jr., H. H. (2014). The Trophic role of a forest salamander: Impacts on invertebrates, leaf litter retention, and the humification process. *Ecosphere, 5*(2), 1-19.

Bürkner, C.P. (2017). Bmrs: an R package for bayesian multilevel models using stan. *Journal of Statistical Software, 80*(1).

Burton, T. M., & Likens, G. E. (1975a). Energy flow and nutrient cycling in salamander populations in the Hubbard Brook Experimental Forest, New Hampshire. *Ecology, 56,* 1068-1080.

Cabe, P. R., Page, R. B., Hanlon, T.J., Aldrich, M.E., Connors, L., & March, D.M. (2007). Fine-scale population differentiation and gene flow in a terrestrial salamander (*Plethodon cinereus*) living in continuous habitat. *Heredity 98***,** 53–60.

Cayuela, H., Quay, L., Dumet, A., Léna, J., Miaud, C., & Rivière, V. (2017). Intensive vehicle traffic impacts morphology and endocrine stress response in a threatened amphibian. *Oryx, 51*(1), 182-188.

Ceballos, G., Ehrlich, P. R., & Dirzo, R. (2017). Biological annihilation via the ongoing sixth mass extinction signaled by vertebrate population losses and declines. *PNAS, 114*(30), E6089-E6096.

Dantzer, B., Fletcher, Q. E., Boonstra, R., & Sheriff, M. J. (2014). Measures of physiological stress: A transparent or opaque window into the status, management and conservation of species. *Conservation Physiology, 2,* 1-18.

Davic, R. D., & Welsh Jr., H. H. (2004). On the ecological roles of salamanders. *Annual Review of Ecology, Evolution, and Systematics, 35,* 405-434.

Gabor, C. R., Davis, D. R., Kim, D. S., Zabierek, K.C., & Bendik, N. F. (2018). Urbanization is associated with elevated corticosterone in Jollyville Plateau salamanders. *Ecological Indicators, 85,* 229-235.

Gade, M., & Peterman, W. (2019). Multiple environmental gradients influence the distribution and abundance of a key forest-health indicator species in the Southern Appalachian Mountains, USA. *Landscape Ecology 34*(3).

Hayes, T. B., Falso, P., Gallipeau, S., & Stice, M. (2010). The cause of global amphibian declines: a Developmental endocrinologist's perspective. *Journal of Experimental Biology, 213*(6), 921–933.

Hesselbarth, M.H.K., Sciaini, M., With, K.A., Wiegand, K., Nowosad, J. 2019. Landscapemetrics: An open-source R tool to calculate landscape metrics. *Ecography, 42*, 1648-1657.

Hoffman, M., Hilton-Taylor, C., Angulo, A., Böhm, M., Brooks, T.M., Butchart, S.H.M., Carpenter, K.E., Chanson, J., Polidoro, B.A., & Sanciangco, J.C. (2010). The impact of conservation on the status of the world's vertebrates. *Science, 330*(6010), 1503-1509.

Hocking, D., & Babbitt, K. (2014). Amphibian contributions to ecosystem services. *Herpetological Conservation and Biology, 9*(1), 1−17.

Homan, R. N., Reed, J. M., & Romero, L. M. (2003). Corticosterone concentrations in free-living spotted salamanders (Ambystoma maculatum). *General and Comparative Endocrinology, 130,* 165-171.

Homan, R. N., Regosin, J.V., Rodrigues, D. M., Reed, M. J., Windmiller, B. S., & Romero, L. M. (2003). Impacts of varying habitat quality on the physiological stress of spotted salamanders. *Animal Conservation, 6*, 11–18.

Homyack, J., Haas, C., & Hopkins, W. (2011). Energetics of surface-active terrestrial salamanders in experimentally harvested forest. *Journal of Wildlife Management, 75*(6), 1367-1278.

Hyde, E.J. &Simons, T.R. (2001). Sampling plethodontid salamanders: sources of variability. *Journal of Wildlife Management, 65*, 624–632.

Jannin, A., Lena, J. P., Deblois, S., & Joly, P. (2012). Use of stress hormonal levels and habitat selection to assess functional connectivity of a landscape for an amphibian. *Conservation Biology, 26*(5), 923-931.

Kaczmarek, J., Kaczmarski, M., & Pędziwiatr, K. (2014). Changes in the batrachofauna in the city of Poznań over 20 years. In: Böhner J, Indykiewicz P (eds.) Urban Fauna. Animal, man, and the City – interactions and relationships. ArtStudio, Bydgoszcz, pp. 169–178.

Kaiser, A., Merckx, T., & Van Dyck, H. (2016). The Urban Heat Island and its spatial scale dependent impact on survival and development in butterflies of different thermal sensitivity. *Ecology and Evolution,* 6(12), 4129–4140.

Konowalik, A., Najbar, A., Konowalik, K., Dylewski, L., Frydlewicz, M., Kisiel, P., Starzecka, A., Zalesna, A., & Kolenda, K. (2020). Amphibians in an urban environment: a case study from a central European city (Wrocław, Poland). *Urban Ecosystems, 23,* 235-243.

Lannoo, M. (2004). *Amphibian declines: The conservation status of United States species*. University of California Press.

Leeson, G. (2018). The Growth, ageing and urbanization of our world. *Journal of Population Ageing, 11* (2), 107-115.

Mccallum. M. L. (2007). Amphibian decline or extinction? Current declines dwarf background extinction rate. *Journal of Herpetology, 41*(3), 483-491.

Merchant, H. C. (1970). Estimated energy budget of the red-backed salamander, *Plethodon cinereus*. Dissertation. *Rutgers University*, Rutgers, New Jersey, USA.

Millikin, A. R., Woodley, S. K., Davis, D. R., & Anderson, J. T. (2019). Habitat characteristics in created vernal pools impact spotted salamander water-borne corticosterone levels. *Wetlands.*

Najbar, B., Szuszkiewicz, E., & Pietruszka, T. (2005). Amphibia in Zielona Góra and the disappearance of their sites located within the administrative borders of the town in the years 1974-2004. *Przegląd Zoologiczny, 49*,155–166.

NLCD 2016 Land Cover (CONUS). (2021). Multi-resolution land characteristics consortium. Retrieved March 25, 2021, from https://www.mrlc.gov/data/nlcd-2016-land-cover-conus.

Novarro, A. J., Gabor, C. R., Goff, C. B., Mezebish, T. D., Thompson, L. M., & Grayson, K. L. (2018). Physiological responses to elevated temperature across the geographic range of a terrestrial salamander. *Journal of Experimental Biology, 221*, 1-10.

Peterman, W., & Semlitsch R. (2013). Fine-scale habitat associations of a terrestrial salamander: The role of environmental gradients and implications for population dynamics. *PLOS ONE 8*(5).

Piironen, J., & Vehtari, A. (2017). Sparsity information and regularization in the horseshoe and other shrinkage. *Electronic Journal of Statistics, 11*(2), 5018-5051.

Reeder, D. M. & Kramer, K. M. (2005). Stress in free-ranging mammals: integrating physiology, ecology, and natural history. *Journal of Mammalogy, 86*(2), 225-235.

Riedel, B.L., Russell, K. R., Ford, W. M., O'Neill. K., & Godwin, H. (2008). Habitat relationships of eastern red-backed salamanders (Plethodon cinereus) in Appalachian agroforestry and grazing systems. *Agriculture Ecosystems & Environment, 124*(34), 229-236.

Rollins-Smith, L A. (2017). Amphibian immunity-stress, disease, and climate change. *Developmental and Comparative Immunology, 66*, 111-119.

Romero, L.M., & Reed, J.M. (2008). Repeatability of baseline corticosterone concentrations. *General and Comparative Endocrinology, 156*, 27–33.

Santymire, R. M., Manjerovic, M. B., & Sacerdote-Velat, A. (2018). A Novel method for the measurement of glucocorticoids in dermal secretions of amphibians. *Conservation Physiology, 6*, 1-12.

Sapolsky, R. M., Romero, L. M., & Munck, A. U. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine Review, 1*, 55-89.

Semlitsch, R. & Bodie, J. (2003). Biological criteria for buffer zones around wetlands and riparian habitats for amphibians and reptiles. *Conservation Biology, 17,* 1219–1228.

Strong, A., Martin, F. L., Jones, K. C., Shore, R. F., & Halsall, C. J. (2017). Subtle effects of environmental stress observed in the early life stages of the Common frog, *Rana temporaria*. *Scientific Reports,7*(1).

Spotila, J. (1972). Role of temperature and water in the ecology of lungless salamanders. *Ecological Monographs*, *42*(1), 95-125.

Stuart, S. N., Chanson, J.S., Cox, N. A., Young, B. E., Rodrigues, A. S. L., Fischman, D. L., & Waller, R.W. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science, 306* (5702), 1783-1786.

Valencia-Aguilar, A., Cortez-Gomez, A. M., & Ruiz-Agudelo, C. A. (2013). Ecosystem services provided by amphibians and reptiles in Neotropical ecosystems. *International Journal of Biodiversity Science, Ecosystem Services and Management, 9*(3).

Vos, C., Verboom, J., Opdam, P., & Ter Braak, C. (2001) Toward ecologically scaled landscape indices. *American Naturalist, 183,* 24–41.

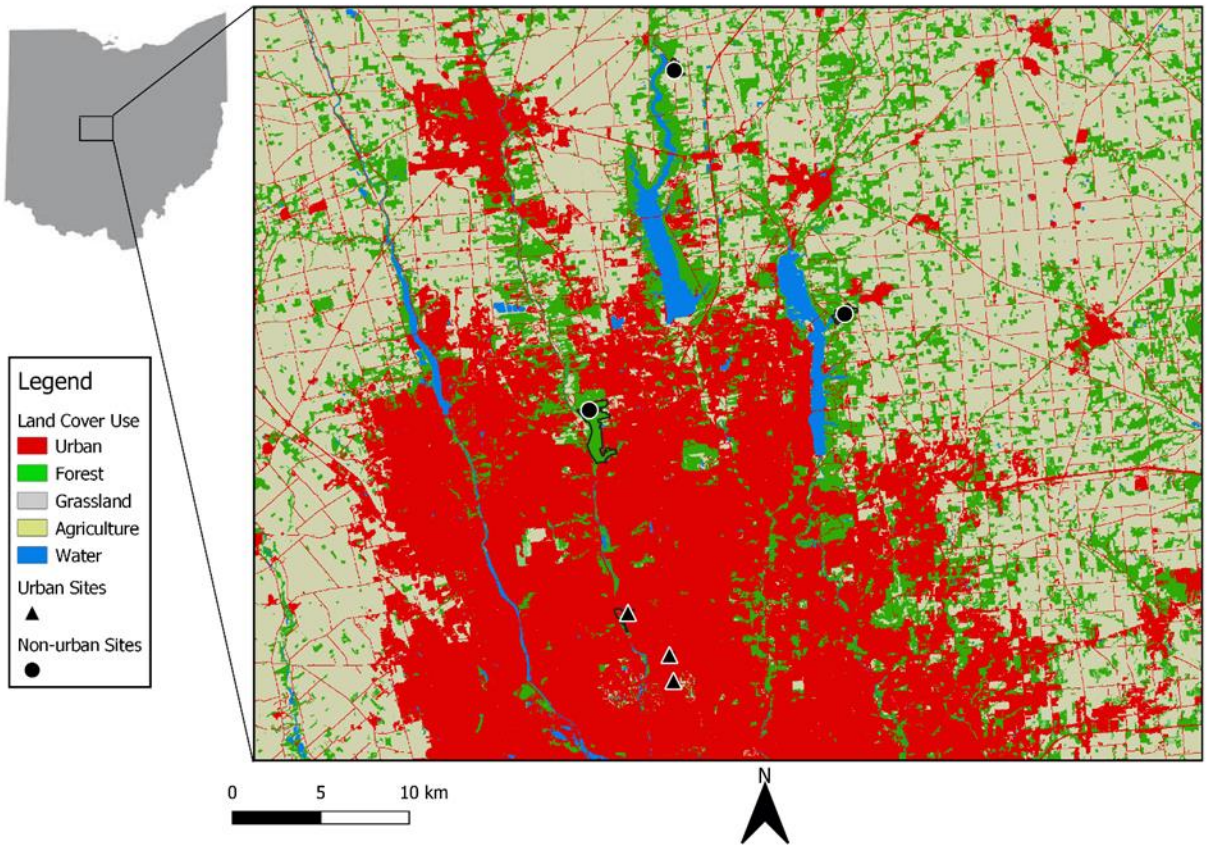
Walton, M. B., Tsatiris, D., & Rivera-Sostre, M. (2006). Salamanders in forest-floor food webs: Invertebrate species composition influences top–down effects. *Pedobiologia, 50*(4), 313-321.

Welsh Jr., H. H. & Droege, S. (2001). A Case for using plethodontid salamanders for monitoring biodiversity and ecosystem integrity of North American forests. *Conservation Biology, 15* (3), 558-569.

Welsh Jr., H. H., & Hodgson, G. R. (2013). Woodland salamanders as metrics of forest ecosystem recovery: A case study from California's Redwoods. *Ecosphere, 4* (5), 1-25.

Wyman, R. (1998). Experiment assessment of salamanders as predators of detrital food webs: Effects on invertebrates, decomposition and the carbon cycle. *Biodiversity and Conservation, 7*, 641-650.

433 Figures and Tables



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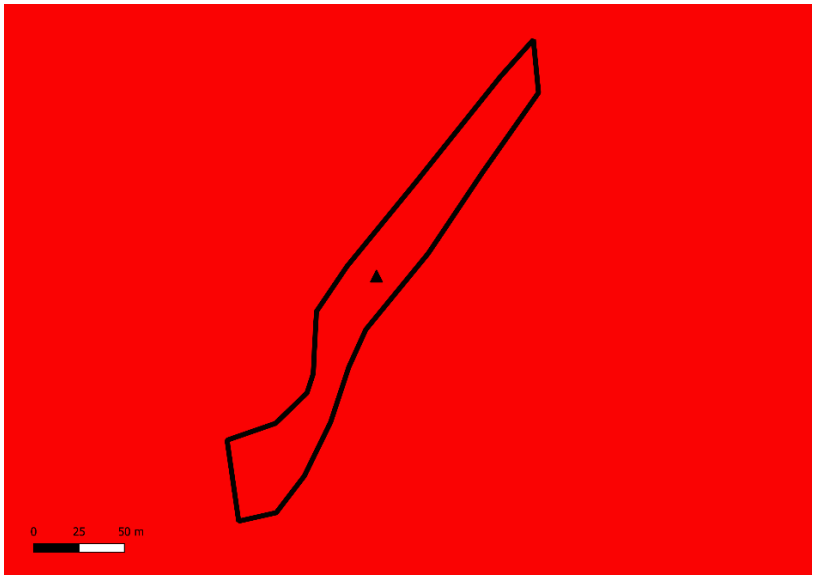

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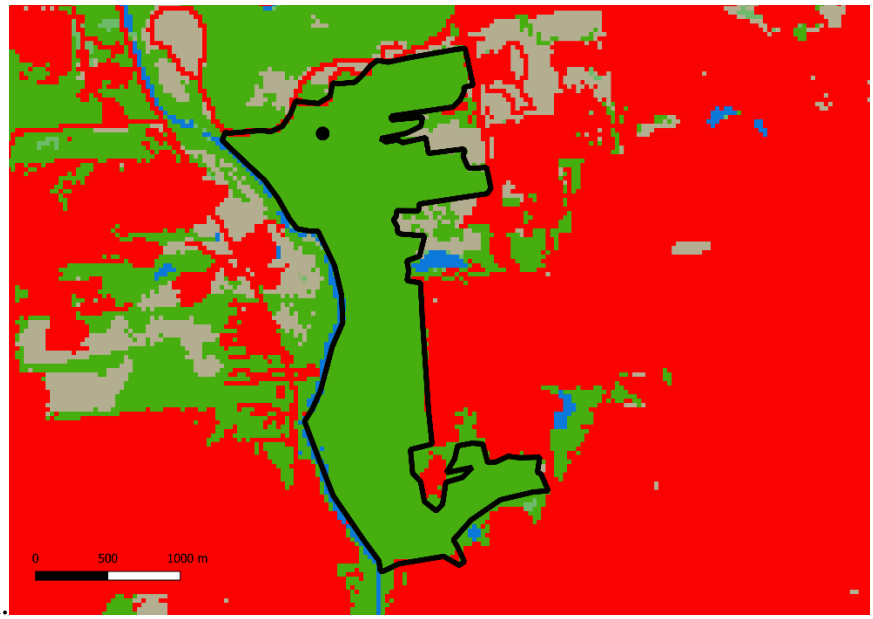
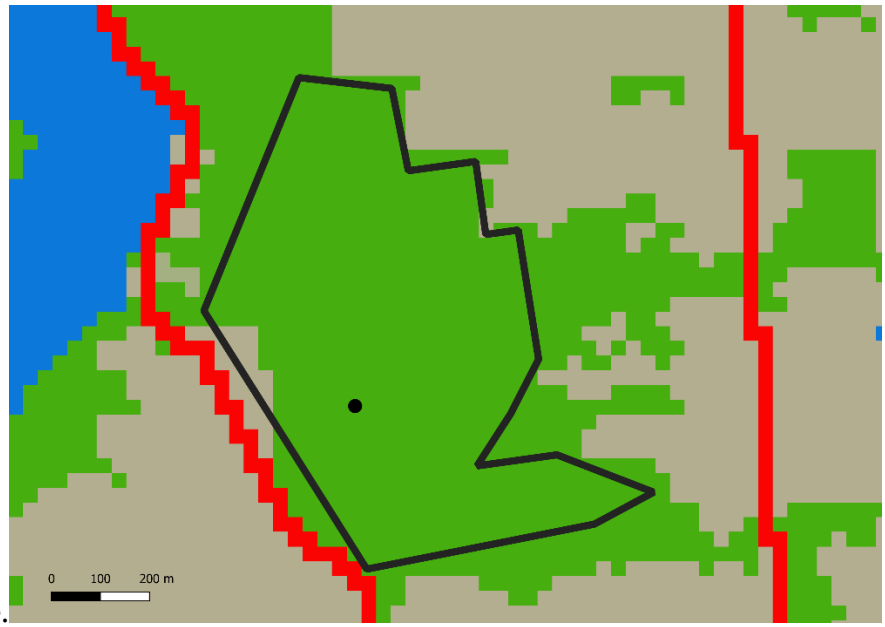
d. 

e. 

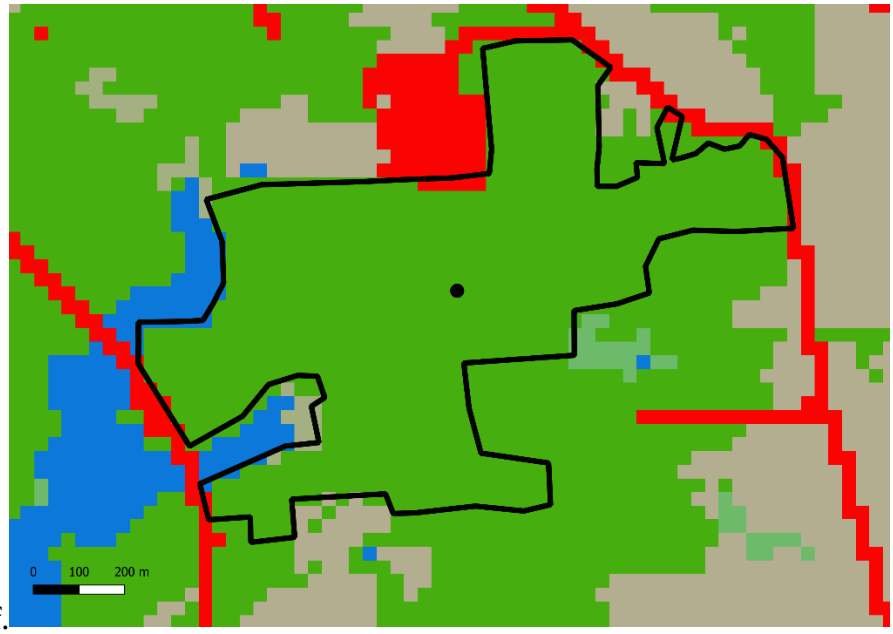
f. 

Figure 1. Landcover classification at each site. Green represents forested land cover and red designates urban land cover. Centroids are placed at the center of the 30 collection points for each park and designated with either a circle (non-urban) or a triangle (urban). The sites in order are a. Iuka Park b. Glenn Echo Park c. Whetstone Park of Roses d. High Banks Metro Park e. Hogback Ridge Park and f. John Beltz Retreat Center. (NLDC 2016 Landcover 2021)

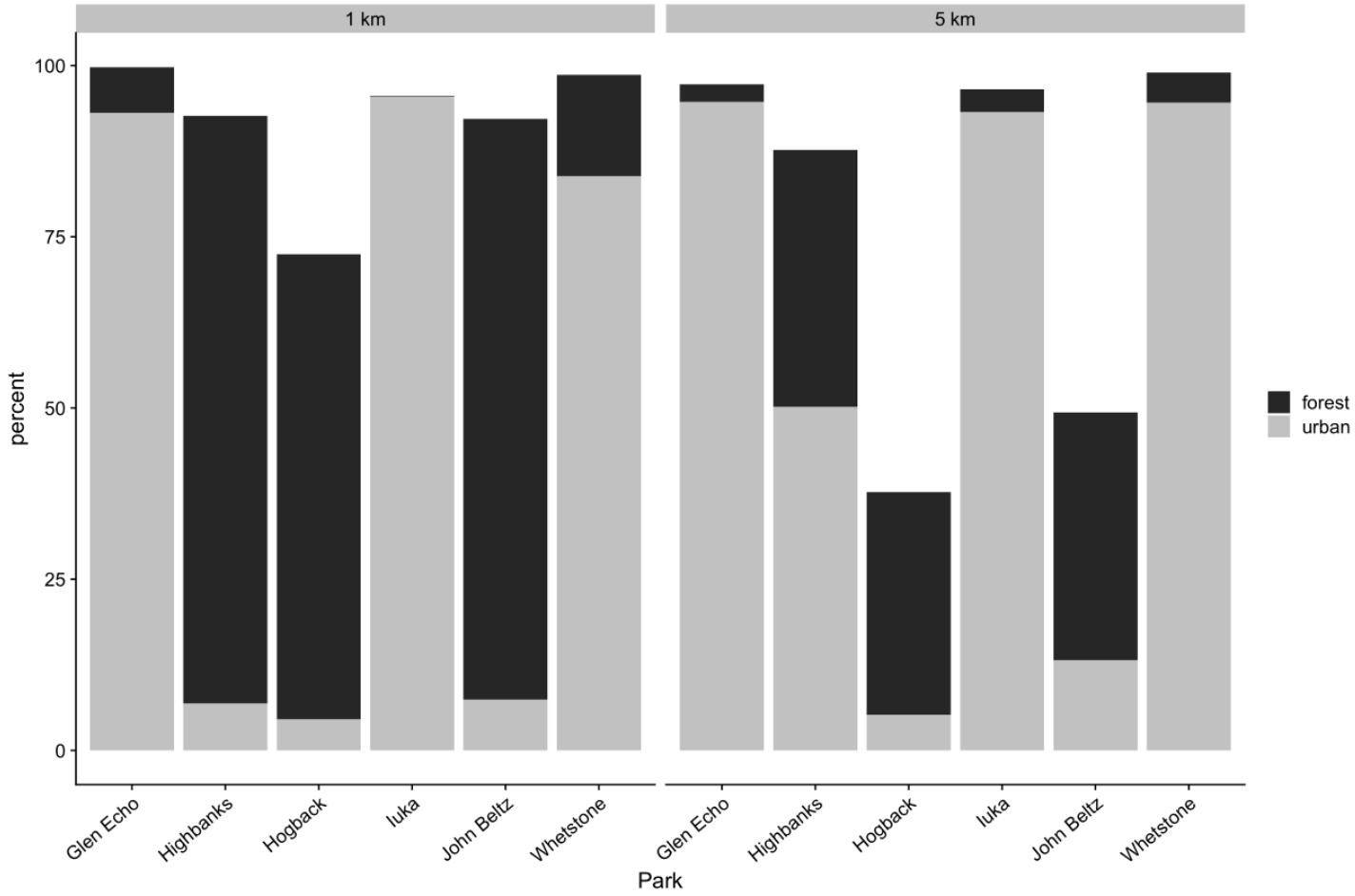


Figure 2. Percentage of each landcover class within a 1km and 5km buffer around the centroid of sampling locations at each site. Data is taken from the NLDC database. The first bar in each set represents the cover at a 1 km buffer and the second bar represents the land cover at a 5 km buffer. The urban sites are Glen Echo, Iuka, and Whetstone and the non-urban sites are Highbanks, Hogback, and John Beltz.

Table 1. Summary of sampled sites. The total site size in represented in hectares and location is the geographic center of all salamander sample locations within a site. Salamander density estimates are from Wilk et al. 2020, which did not sample Hogback Ridge.

Site	**Site size (ha)**	**Centroid Coordinates**	**Salamander Density (per m2)**	**Site Designation**
Iuka	1.6	-82.99958, 40.00597	3.51	Urban

Glen Echo	1.7	-82.99990,	40.01912	0.59	Urban
Whetstone	55.0	-83.02351,	40.04307	3.27	Urban
High Banks	469.0	-83.03067,	40.14689	2.01	Non-urban
John Beltz	37.5	-82.85250,	40.17724	3.85	Non-urban
Hogback Ridge	16.6	-82.94328,	40.31077	unknown	Non-urban

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462 Table 2. Mean (± SD) of dermal CORT excretion (pg/swab) standardized by SVL in *Plethodon*
463 *cinereus* collected at six sites. Mean (± SD) of dermal CORT of all three non-urban and all three
464 urban sites.

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Site	Date Sampled	Urban/Non-urban	Avg CORT	Std Dev	Avg SVL	Std Dev
Glen Echo Park	10/14/2020	Urban	458.98	321.17	3.78	0.41
Iuka Park	10/7/2020	Urban	371.71	324.75	3.68	0.51
Whetstone Park of Roses	10/13/2020	Urban	300.47	139.35	3.92	0.50
Avg All Urban Sites			377.05	64.82	3.52	0.55
Highbanks Metro Park	10/6/2020	Non-urban	387.10	173.50	3.44	0.65
Hogback Ridge Park	10/16/2020	Non-urban	657.95	540.22	3.50	0.44
John Beltz Retreat Center	10/10/2020	Non-urban	591.80	426.37	3.62	0.51
Avg All Non-urban Sites			545.62	153.25	3.79	0.48

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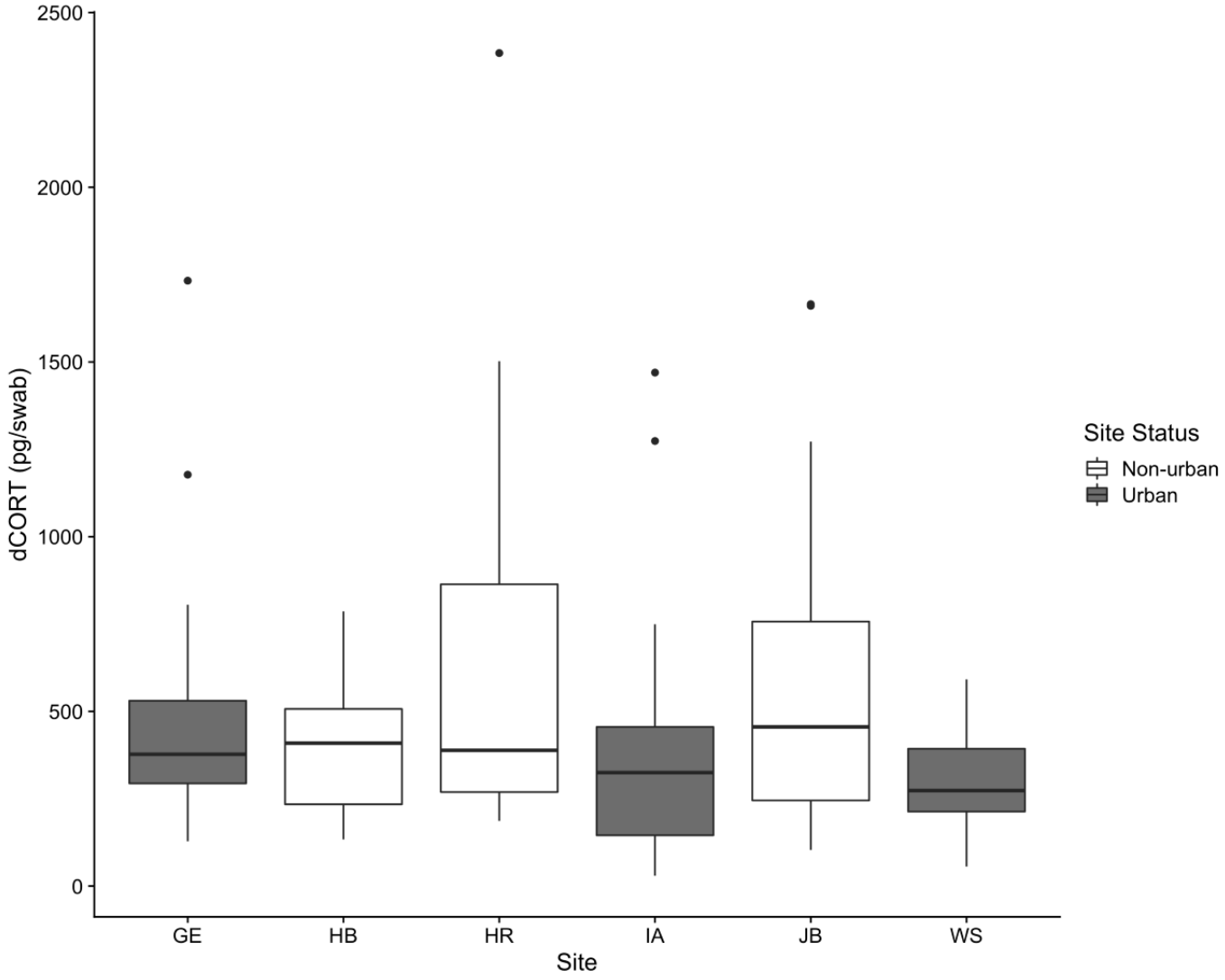
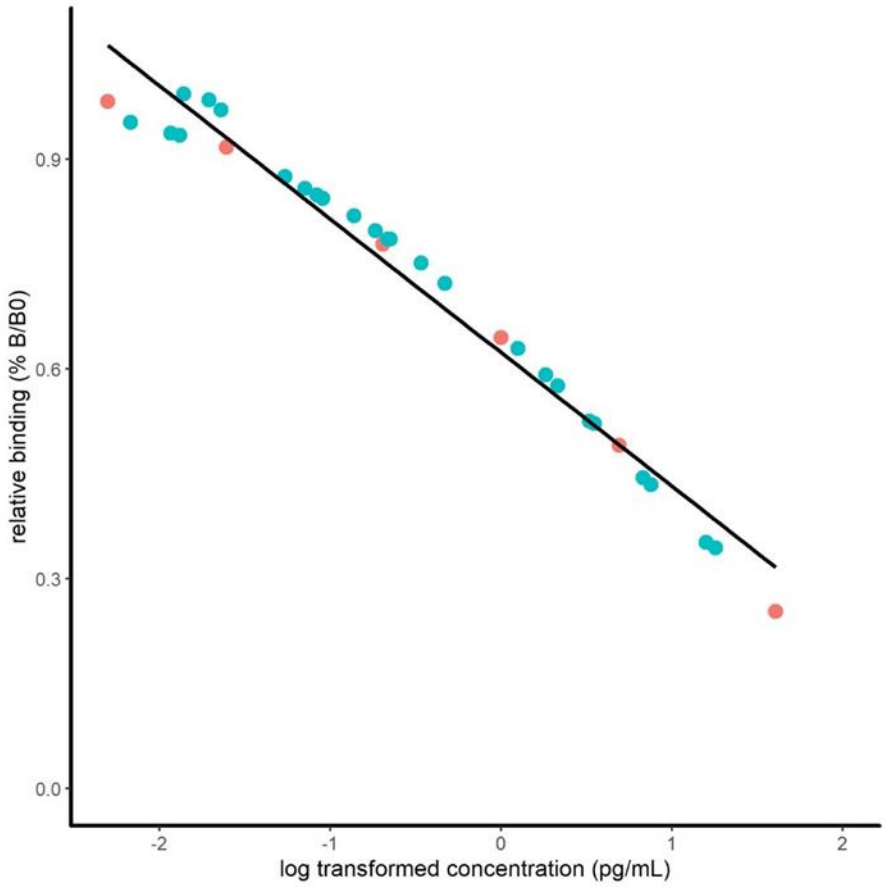


Figure 3. Distribution of dCORT (pg/swab standardized by SVL) levels. The three sites designated as non-urban are Highbanks Metro Park (HB), Hogback Ridge Park (HR), and John-Beltz retreat center (JB). The urban sites are Glen Echo Park (GE), Iuka Park (IA), and Whetstone Park of Roses (WS).



Supplementary Figure 1. Relative binding in relation to log transformed dCORT concentration (pg/mL) for serially diluted standards (red points) and salamander swab samples from nine individuals (blue points).

Appendix

Table 1. Percent soil moisture at each site after samples were dried for 24 hours.

Site	Designation	Wet Weight	Dry Weight	Percent Soil Moisture
Glen Echo	Urban	197.56	172.67	12.6%
Hogback	Non-urban	135.88	119.33	12.2%
Highbanks	Non-urban	122.19	108.91	10.9%
Iuka Ravine	Urban	90	76.46	15.0%
Whetstone	Urban	89.99	73	18.9%
John Beltz	Non-urban	31.71	30.15	4.9%

Table 2. Averages and standard deviations from each park of the soil temperature (°C) under a cover object (STU), surface soil temperature under a cover object (SSTU), a random soil temperature near the collection site (ST_R), and a random surface soil temperature near the collection site (SSTR). Urban sites are Iuka, Whetstone, and Glen Echo and non-urban sites are Highbanks, John Beltz, and Hogback Ridge.

Site	Avg STU ± SD	Avg SSTU ± SD	Avg STR ± SD	Avg SSTR ± SD	Number of Samples
Highbanks	12.3 ± 0.60	9.8 ± 1.3	12.1 ± 0.52	10.7 ± 1.7	30
Iuka	15.5 ± 0.42	14.5 ± 1.6	15.5 ± 0.51	15.7 ± 2.3	30
John Beltz	16.5 ± 0.29	16.7 ± 1.6	16.7 ± 0.35	18.2 ± 1.0	30
Whetstone	16.1 ± 1.6	12.1 ± 2.2	16.3 ± 0.56	11.8 ± 2.6	30
Glen Echo	15.1 ± 0.66	10.9 ± 2.5	14.7 ± 1.0	10.4 ± 1.1	30
Hogback Ridge	13.1 ± 0.49	7.2 ± 0.98	13.2 ± 0.43	7.3 ± 2.2	30